CompCytogen 13(4): 435–449 (2019)
doi: 10.3897/CompCytogen.v13i4.48368
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Linking karyotypes with DNA barcodes: proposal for a new standard in chromosomal analysis with an example based on the study of Neotropical Nymphalidae (Lepidoptera)

Vladimir A. Lukhtanov[1,2], Yaroslavna Iashenkova[2,3]

1 *Department of Karyosystematics, Zoological Institute of the Russian Academy of Sciences, Universitetskaya emb. 1, St. Petersburg 199034, Russia* **2** *Department of Entomology, St. Petersburg State University, Universitetskaya emb. 7/9, St. Petersburg 199034, Russia* **3** *Department of Genetics and Biotechnology, St. Petersburg State University, Universitetskaya emb. 7/9, St. Petersburg 199034, Russia*

Corresponding author: *Vladimir A. Lukhtanov* (lukhtanov@mail.ru)

Academic editor: *V. G. Kuznetsova* | Received 11 November 2019 | Accepted 4 December 2019 | Published 17 December 2019

http://zoobank.org/911CBEA6-C1C8-4D68-81E2-ECB5B61A5706

Citation: Lukhtanov VA, Iashenkova Y (2019) Linking karyotypes with DNA barcodes: proposal for a new standard in chromosomal analysis with an example based on the study of Neotropical Nymphalidae (Lepidoptera). Comparative Cytogenetics 13(4): 435–449. https://doi.org/10.3897/CompCytogen.v13i4.48368

Abstract

Chromosomal data are important for taxonomists, cytogeneticists and evolutionary biologists; however, the value of these data decreases sharply if they are obtained for individuals with inaccurate species identification or unclear species identity. To avoid this problem, here we suggest linking each karyotyped sample with its DNA barcode, photograph and precise geographic data, providing an opportunity for unambiguous identification of described taxa and for delimitation of undescribed species. Using this approach, we present new data on chromosome number diversity in neotropical butterflies of the subfamily Biblidinae (genus *Vila* Kirby, 1871) and the tribe Ithomiini (genera *Oleria* Hübner, 1816, *Ithomia* Hübner, 1816, *Godyris* Boisduval, 1870, *Hypothyris* Hübner, 1821, *Napeogenes* Bates, 1862, *Pseudoscada* Godman et Salvin, 1879 and *Hyposcada* Godman et Salvin, 1879). Combining new and previously published data we show that the species complex *Oleria onega* (Hewitson, [1852]) includes three discrete chromosomal clusters (with haploid chromosome numbers n = 15, n = 22 and n = 30) and at least four DNA barcode clusters. Then we discuss how the incomplete connection between these chromosomal and molecular data (karyotypes and DNA barcodes were obtained for different sets of individuals) complicates the taxonomic interpretation of the discovered clusters.

Copyright *V.A. Lukhtanov, Y. Iashenkova.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Keywords
karyotype, DNA barcoding, *COI*, meiosis, metaphase, Lepidoptera, Nymphalidae, Biblidinae, Danainae, Ithomiini, Peru

Introduction

Chromosomal data are an important source of information for taxonomic, evolutionary and comparative phylogenetic studies (White 1973). However, the application of these data is often difficult because of unclear taxonomic identity (e.g. Petrova et al. 2015) or doubtful species identification (or even due to the lack of a species identification) of the samples that were used as vouchers for karyotype analysis [e.g. some samples and identifications in Robinson (1971) and Brown et al. (2004)]. Theoretically, one can try to find these samples, provided that they were neatly labeled and can be recognized, are stored in accessible museums and have not been lost, and then check their identification using taxonomic literature or comparison with type specimens. However, it is complicated and almost impossible in practice.

To avoid this problem, here we suggest linking each karyotyped sample with its DNA barcode. It was empirically demonstrated that the mitochondrial DNA barcode, a relatively short fragment of the mitochondrial *COI* gene (658 base pairs) (i.e., a negligible part of the genome in terms of size), could differentiate up to 95% of species in many taxa (Hebert et al. 2003, 2004; Hebert and Gregory 2005; Hajibabaei et al. 2006; Lukhtanov et al. 2009). In addition, the barcoding DNA protocol provides a standardized system for storing information on vouchers that served as the basis for DNA barcoding, including the image, the exact label and the storage location of the samples. This makes it possible, if necessary, to relatively easily find and re-examine a voucher.

Obtaining barcodes is currently a simple technical task, which can be carried out in almost any laboratory or on a commercial basis. Our personal experience, based on a molecular analysis of the fauna of Central Asia, Eastern Europe and Western Asia (Lukhtanov et al. 2009, 2016; Lukhtanov 2017; Pazhenkova and Lukhtanov 2019), shows that if there are barcode libraries (Ward et al. 2009; Dincă et al. 2011) for a given region and for a given taxonomic group, barcodes ensure almost 100% success of species identification. Even if such a library is not currently available for a group or region under study, the presence of a barcode makes it possible to reliably identify the sample in the future. Thus, linking karyotypes with DNA barcodes resolves the problem of reliable species identification.

Additionally, combination of DNA barcodes and karyotypes represents a powerful tool for detection, delimitation and description of unrecognized species (Lukhtanov et al. 2015; Vishnevskaya et al. 2016, 2018). Therefore, linking karyotypes with DNA barcodes, potentially resolves the problem of unclear species identity in chromosomal studies.

The approach based on combination of chromosomal and DNA barcode data has been already used in different studies on butterflies (Lukhtanov et al. 2014, 2015;

Lukhtanov and Dantchenko 2017), fish (Marques et al. 2013), lizards (de Matos et al. 2016), mammals (Tavares et al. 2015) and mussels (Garcia-Souto et al. 2017). However, its principles have not been explicitly formulated.

In this paper, we demonstrate the algorithm, features and capabilities of the proposed approach with the butterflies of the Neotropical fauna.

Material and methods

Samples

The samples were collected in Peru in 2013 by V.A.Lukhtanov. The information on localities where the specimens were collected is presented in the Table 1. The morphology-based species identification was carried out by comparing the specimens with butterfly images figured at Butterflies of America site (https://www.butterfliesofamerica.

Table 1. List of the samples of the genera *Oleria* Hübner, 1816, *Ithomia* Hübner, 1816, *Vila* Kirby, 1871, *Pseudoscada* Godman et Salvin, 1879, *Godyris* Boisduval, 1870, *Hypothyris* Hübner, 1821, *Napeogenes* Bates, 1862 and *Hyposcada* Godman et Salvin, 1879 collected by V.A.Lukhtanov and used in the study.

Id	BOLD Id	Genus	Species	N	Exact site	Latitude / Longitude	Altitude	Collection date
A107	NOB001-17	*Oleria*	*didymaea ramona*	n = 22	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A108	NOB002-17	*Ithomia*	*salapia*	n = 34	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A111	NOB004-17	*Vila*	*emilia*	n = 30	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A112	NOB005-17	*Vila*	*emilia*	–	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A113	NOB006-17	*Vila*	*emilia*	n = 30	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A115	NOB007-17	*Vila*	*emilia*	n = 30	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A121	NOB008-17	*Oleria*	*gunilla serdolis*	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A122	NOB009-17	*Oleria*	*gunilla serdolis*	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A123	NOB010-17	*Oleria*	*gunilla serdolis*)	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A125	NOB011-17	*Oleria*	*onega*	n = 15	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A127	NOB012-17	*Oleria*	*gunilla serdolis*	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A124	NOB013-17	*Oleria*	*onega*	n = 15	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A129	n/a	*Pseudoscada*	*timna*	n = 15	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A130	NOB014-17	*Ithomia*	*salapia*	n = 34	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A131	NOB015-17	*Godyris*	*zavaleta*	n = 33,35	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A132	NOB016-17	*Ithomia*	*salapia*	n = 35	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A133	NOB017-17	*Ithomia*	*salapia*	n = 36	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A135	NOB018-17	*Ithomia*	*salapia*	n = 36	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A136	NOB019-17	*Hypothyris*	*euclea*	n = 14	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A137	NOB020-17	*Napeogenes*	*sylphis*	n = 14	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A140	NOB021-17	*Hyposcada*	*kena*	n = 14	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A141	NOB022-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A142	NOB023-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A143	NOB024-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A144	NOB025-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A145	NOB026-17	*Godyris*	*dircenna*	n = 36	Cayumba	09°29'43"S, 75°58'01"W	786 m	6 September 2013

com/L/Nymphalidae.htm). Photographs of all specimens used in the analysis, as well as collecting data, are available on the Barcode of Life Data System (BOLD) at http://www.boldsystems.org/. The specimens are deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia.

Chromosomal analysis

Gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3–36 months at +4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at +18–20 °C. Karyotypes (Figs 1–19) were analyzed as previously described (Przybyłowicz et al. 2014; Lukhtanov and Shapoval 2017). Briefly, the stained testes were placed in a drop of 40% lactic acid on a slide, and spermatocysts were dissected from gonad membranes using entomological pins before covering everything with a coverslip. Different degrees of chromosome spreading were observed by gradually increasing the pressure on the coverslip. Haploid chromosome numbers (n) were counted at meiotic metaphase I (MI) and metaphase II (MII).

DNA barcoding

Standard *COI* barcodes (658-bp 5' segment of mitochondrial cytochrome oxidase subunit I) were studied. Legs were sampled from the karyotyped specimens, and sequence data from the DNA barcode region of *COI* were obtained at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using primers and protocols described in Hajibabaei et al. (2005), Ivanova et al. (2006) and deWaard et al. (2008).

The DNA-barcode-based species identification was carried out by using the BOLDSYSTEMS Identification Engine (http://www.boldsystems.org/index.php/IDS_OpenIdEngine).

The Bayesian majority rule consensus tree of the analyzed samples (Figs 20, 21) was constructed as previously described (Sahoo et al. 2016; Lukhtanov 2017; Lukhtanov and Dantchenko 2017) using the sequences obtained in this study as well as the published sequences uploaded from GenBank (de-Silva et al. 2010). Briefly, sequences were aligned using the BioEdit software (Hall 1999) and edited manually. The Bayesian analysis was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burnin = 0.25, nst = 6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. The consensus of the obtained trees was visualised using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Karyotypes
Subfamily Biblidinae

***Vila emilia* (Cramer, 1779)**
Fig. 1

The meiotic karyotype was found to include 30 bivalents of similar size.

Subfamily Danainae
Tribe Ithomiini

***Oleria didymaea ramona* (Haensch, 1909)**
Fig. 2

The meiotic karyotype was found to include 22 bivalents of similar size.

***Ithomia salapia* Hewitson, [1853]**
Figs 3–6

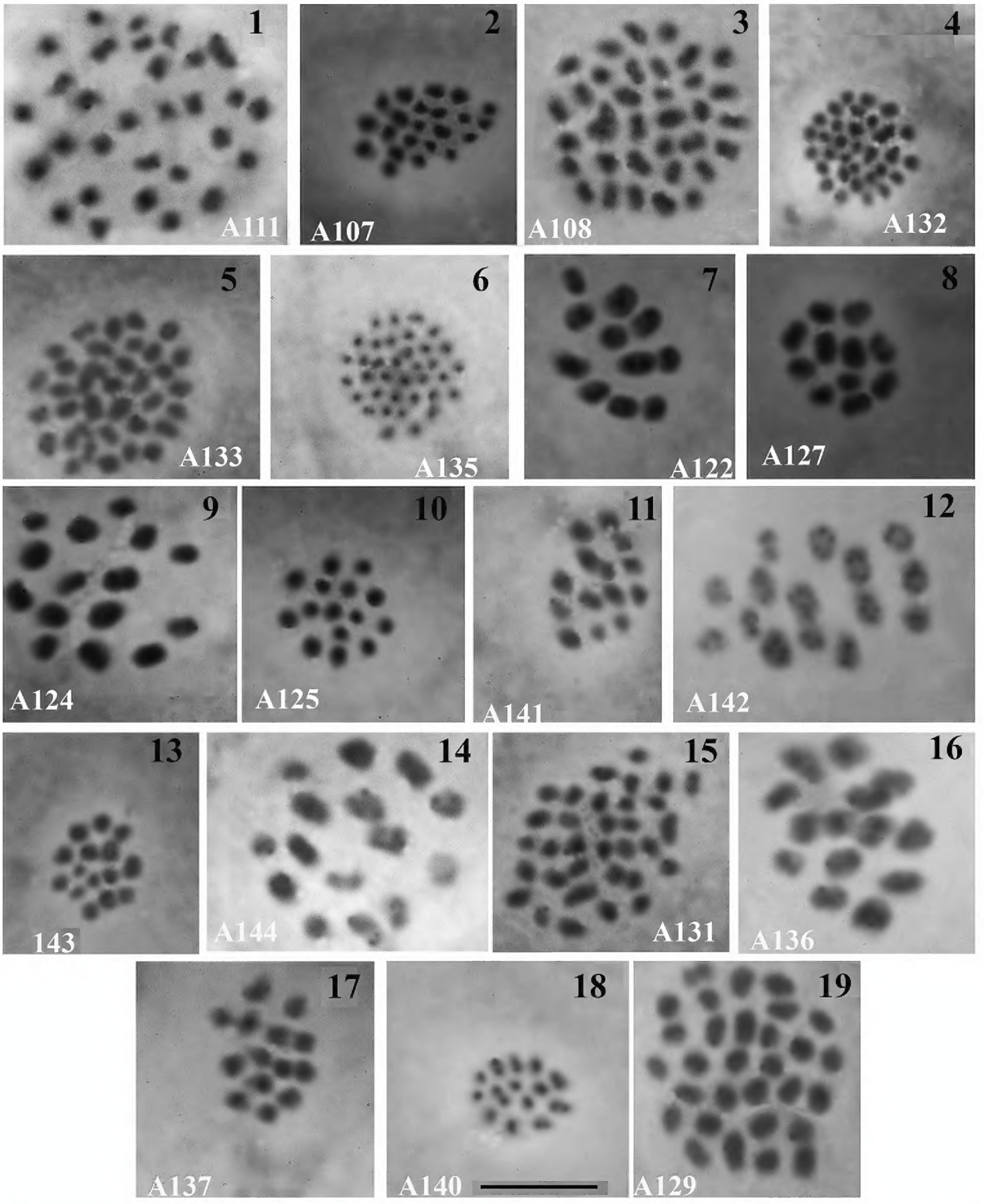
The meiotic karyotype was found to include 34 bivalents in a single studied specimen from Puente Itaya (Peru, 60 km SSW Ikitos). One bivalent was slightly larger than the rest ones. The meiotic karyotype was found to include 35–36 bivalents of similar size in the specimens from Tingo Maria.

***Oleria gunilla serdolis* (Haensch, 1909)**
Figs 7, 8

The meiotic karyotype was found to include 11 bivalents. Two bivalents were larger than the other nine ones.

***Oleria onega* (Hewitson, [1852])**
Figs 9–14

The meiotic karyotype was found to include 15 bivalents. The bivalents had different sizes and shapes.



Figures 1–19. Male metaphase I (MI) and II (MII) plates of Ithomiini and Biblidinae **1** A111, *Vila emilia*, MI, n = 30 **2** A107, *Oleria didymaea ramona*, MI, n = 22 **3** A108, *Ithomia salapia*, MI, n = 34 **4** A132, *Ithomia salapia*, MII, n = 35 **5** A133, *Ithomia salapia*, MI, n = 36 **6** A135, *Ithomia salapia*, MII, n = 36 7 **8** A122 *Oleria gunilla serdolis*, MI, n = 11 **9** A124, *Oleria onega*, MI, n = 15 **10** A125, *Oleria onega*, MII, n = 15 **11** A141, *Oleria onega*, MI, n = 15 **12** A142, *Oleria onega*, MI, n = 15 **13** A143, *Oleria onega*, MII, n = 15 **14** A144, *Oleria onega*, MI, n = 15 **15** A131, *Godyris zavaleta*, MI, n = 33 **16** A136, *Hypothyris euclea*, MI, n = 14 **17** A137, *Napeogenes sylphis*, MI, n = 14 **18** A140, *Hyposcada kena*, MII, n = 14 **19** A129, *Pseudoscada timna*, MI, n = 30. Scale bar: 10 μ in all figures.

***Godyris zavaleta* (Hewitson, [1855])**
Fig. 15

The meiotic karyotype was found to include cells with 33 and 35 chromosomal elements, presumably bivalents. 34 bivalents were counted in a single studied specimen from Tingo Maria.

***Hypothyris euclea* (Godart, 1819)**
Fig. 16

The meiotic karyotype was found to include 14 bivalents of similar size.

***Napeogenes sylphis* (Guérin-Méneville, [1844])**
Fig. 17

The meiotic karyotype was found to include 14 bivalents of similar size.

***Hyposcada kena* (Hewitson, 1872)**
Fig. 18

The meiotic karyotype was found to include 14 bivalents. The bivalents had different sizes and shapes.

***Pseudoscada timna* (Hewitson, [1855])**
Fig. 19

The meiotic karyotype was found to include 30 bivalents of similar size. The bivalents formed a gradient size row.

DNA barcodes

All studied species were found to be significantly differentiated with respect to the DNA barcode region and formed distinct clusters on the BI tree (Fig. 20). However, if additional sequences from GenBank were added, the picture became more intricate. Particularly, *Oleria onega* was found to have very complicated structure with numerous differentiated haplotypes forming three monophyletic and one paraphyletic clusters

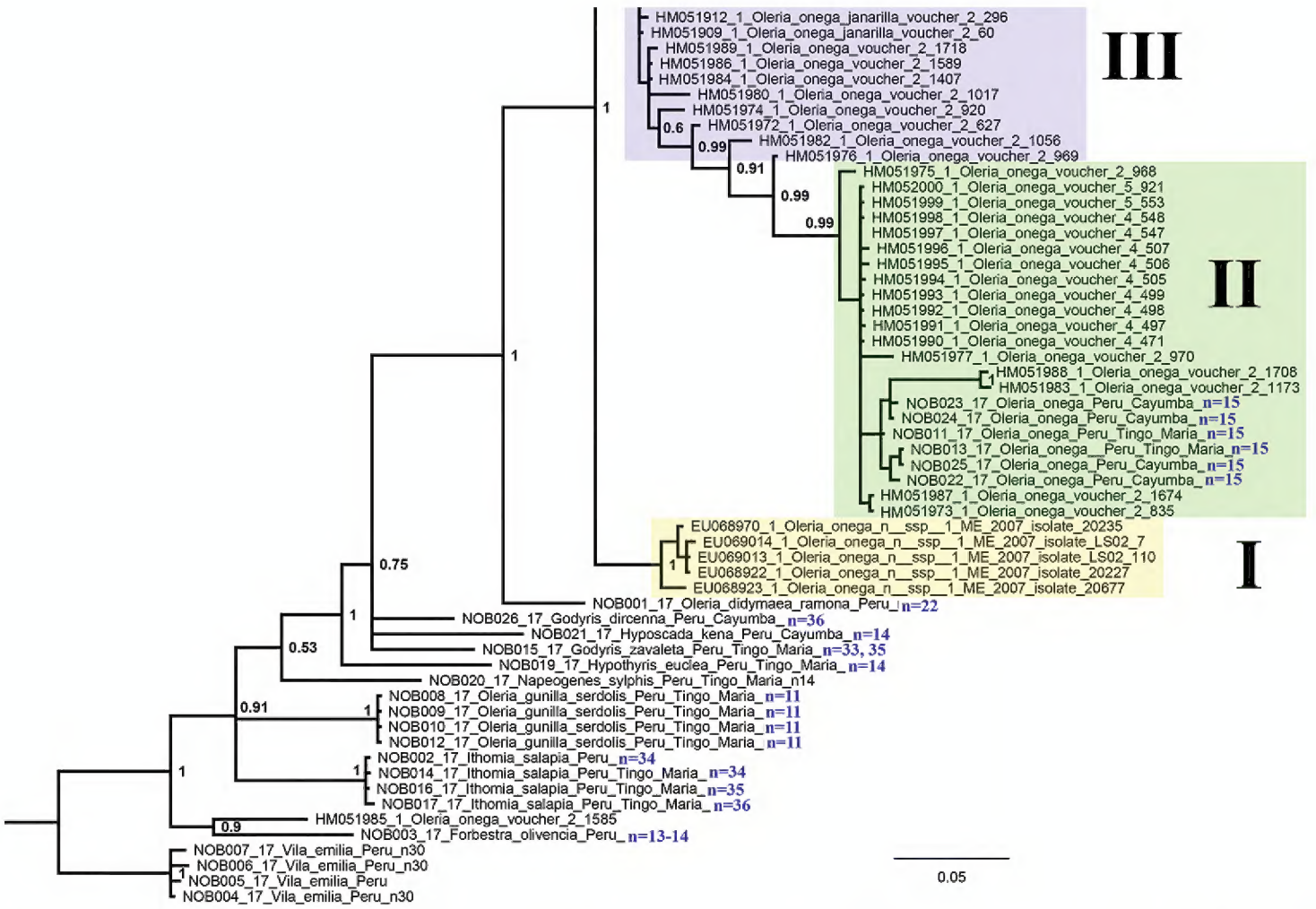


Figure 20. Fragment of the Bayesian majority rule consensus tree of the analyzed samples of Ithomiini inferred from *COI* sequences. I, II and III are the recovered clusters of the *Oleria onega* species complex (see Fig. 21 for the complete structure of the cluster III and the cluster IV). Haploid chromosome numbers (n) are shown after the tip labels. *Vila emilia* (subfamily Biblidinae) was used to root the tree. Bayesian posterior probabilities higher than 0.5 are shown next to the recovered branches.

(Figs 20, 21). The karyotyped samples of this species with the chromosome number n = 15 were found to belong to the cluster II.

Discussion

The Neotropics is one of the most species-rich regions of the world, and the nymphalids are the most speciose butterfly family (Van Nieukerken et al. 2011). Therefore, it is not surprising that the neotropical fauna of Nymphalidae is very rich in species (site (https://www.butterfliesofamerica.com/L/Nymphalidae.htm).

Chromosomal studies represent only a small part of the Neotropical nymphalid diversity (de Lesse 1967, 1970a, b; de Lesse and Brown 1971; Wesley and Emmel 1975; Suomalainen and Brown 1984; Brown et al. 1992, 2004, 2007a, b; McClure et al. 2017; Lukhtanov 2019a). However, they demonstrate an extremely high level of the interspecific karyotype variation and a potential for solving taxonomic problems within the South American nymphalid species. This potential is practically not used (but see: Suomalainen and Brown 1984; Constantino and Salazar 2010; McClure et al. 2017) in opposite to the numerous chromosomally based taxonomic studies in

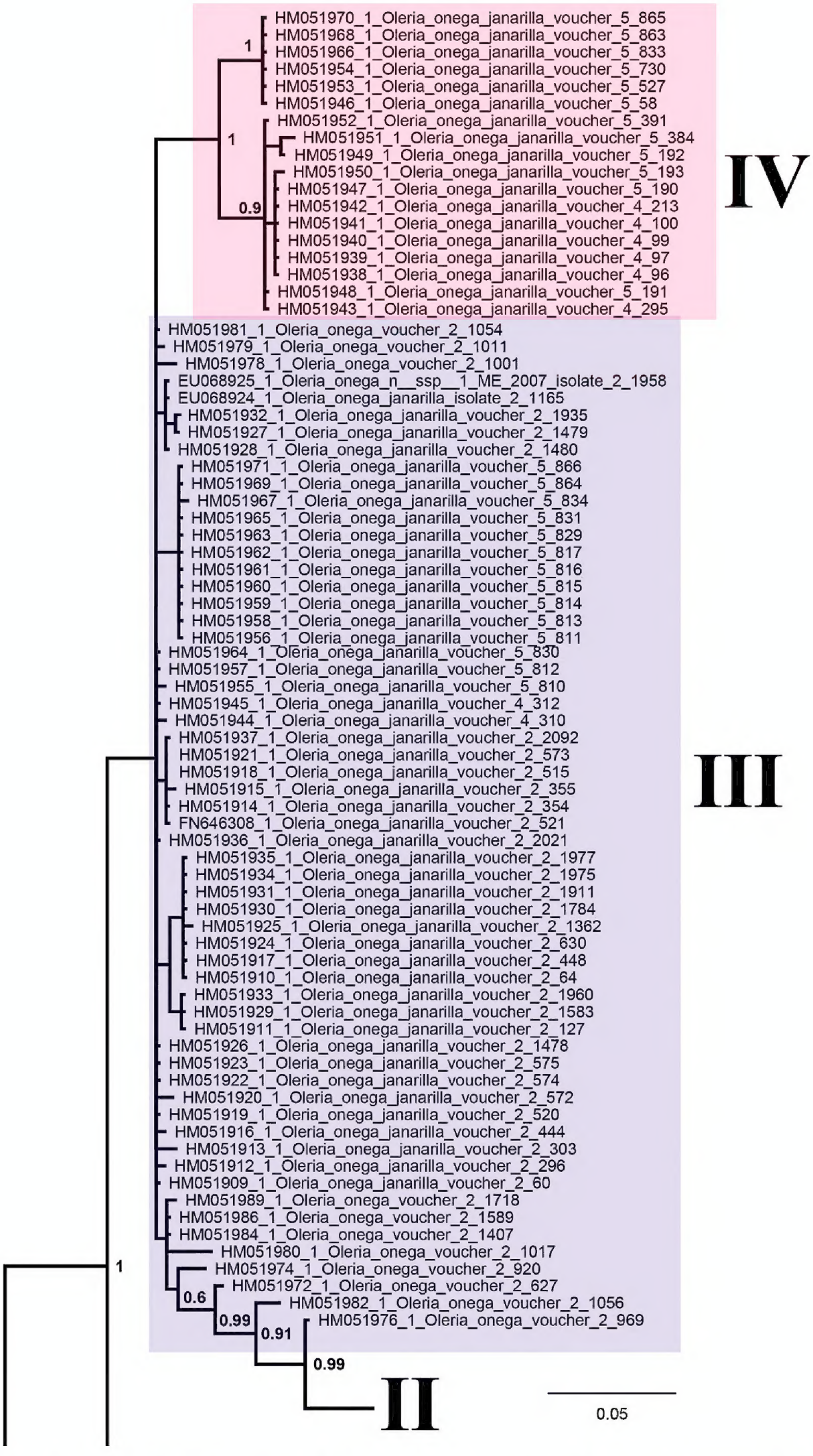


Figure 21. Fragment of the Bayesian majority rule consensus tree of the analyzed samples of Ithomiini inferred from *COI* sequences. The clusters III and IV of the *Oleria onega* species complex are shown. Bayesian posterior probabilities higher than 0.5 are shown next to the recovered branches.

palearctic butterflies (Lorković 1958; de Lesse 1960; Lukhtanov et al. 2011, 2015; Talavera et al. 2013).

In this study we suggest a plan for further analysis of the Neotropical Nymphalidae based on a parallel analysis of chromosomal and molecular markers.

Using this approach, we confirm the previously published data on the karyotypes of *Godyris dircenna* (n = 36), *Hypothyris euclea* (n = 14), *Napeogenes sylphis* (n = 14) and *Oleria gunilla* (n = 11) (Brown et al. 2004).

Haploid chromosome number n=30 is found by us in *Pseudoscada timna*, whereas n = 31 was reported for this taxon by Brown et al. (2004).

We provide the first data on karyotypes of *Vila emilia* and demonstrate a high interspecific chromosome number variation in this genus (previously n = 15 was reported for an unidentified *Vila* species from western Brazil; Brown et al. 2007a).

We show chromosome number n = 14 for *Hyposcada kena* confirming high level of interspecific variation in the genus *Hyposcada* (from n = 12 to n = 19) (Brown et al. 2004).

Different chromosome numbers were previously reported for *Godyris zavaleta* by Brown et al. (2004): n = 46 (on the page 220–221), n = 35–45 (p. 222), n = 36–46 (p. 224), n = 40 (p. 229). However, the credibility and the reason for this variation were not discussed. We provide n = 33 for this species and point out the need for further study of this taxon.

Even more interesting data were obtained regarding the species *Oleria didymaea* (Hewitson, 1876) and *O. onega*. We found n = 22 in the taxon identified by us as *Oleria didymaea ramona* (Haensch, 1909), whereas n= 15 was reported for taxon identified as *Oleria alexina didymaea* (Brown et al. 2004) raising the question of further study of the complex *Oleria didymaea – alexina.*

Based on chromosome numbers, we hypothesize that *Oleria onega* is a complex of at least three species with different chromosome numbers: n = 15 (our data), n = 22 and n = 30 (Brown et al. 2004). A similar conclusion can be made on the basis of molecular data that show the presence of at least four clusters of DNA barcodes in this complex (Figs 20, 21). The status of the detected chromosomal races and mitochondrial clusters could be theoretically resolved based on analysis of: (1) congruence of chromosomal and molecular characters in different sets of individuals, or (2) pattern imitating (vs not imitating) linkage of chromosomal and mitochondrial markers that are known to be unlinked (Lukhtanov at al 2015; Vishnevskaya et al. 2016, 2018; Lukhtanov 2019b). Unfortunately, the previously karyotyped samples (Brown et al. 2004) were not studied with respect to molecular markers, and vice versa, the vouchers for molecular studies were not karyotyped (de-Silva et al. 2010).

The incomplete connection between the chromosomal and molecular data (karyotypes and DNA barcodes were obtained for different sets of individuals) complicates the taxonomic interpretation of the discovered clusters. Nevertheless, we predict that in future linking karyotypes with DNA barcodes will result in a significant rearrangement of taxonomy of the genus *Oleria*.

Acknowledgements

We thank Fedor Konstantinov (St. Petersburg State University) for help in butterfly collecting and research. The study was supported by the projects RFBR 18-04-00263-a, RFBR 17-04-00828-a and state research project AAAA-A19-119020790106-0.

References

BOLD Systems Identification (2019) BOLD Systems Identification. http://boldsystems.org/index.php/IDS_OpenIdEngine

Brown KS, Emmel TC, Eliazar PJ, Suomalainen E (1992) Evolutionary patterns in chromosome numbers in neotropical Lepidoptera I. Chromosomes of the Heliconiini (Family Nymphalidae: Subfamily Nymphalinae). Hereditas 117: 109–125. https://doi.org/10.1111/j.1601-5223.1992.tb00165.x

Brown KS, von Schoultz B, Suomalainen E (2004) Chromosome evolution in Neotropical Danainae and Ithomiinae (Lepidoptera). Hereditas 141: 216–236. https://doi.org/10.1111/j.1601-5223.2004.01868.x

Brown KS, Freitas AVL, Wahlberg N, von Schoultz B, Saura AO, Saura A (2007a) Chromosomal evolution in the South American Nymphalidae. Hereditas 144: 137–148. https://doi.org/10.1111/j.2007.0018-0661.02015.x

Brown KS, Freitas AVL, von Schoultz B, Saura AO, Saura A (2007b) Chromosomal evolution of South American frugivorous butterflies in the Satyroid clade (Nymphalidae: Charaxinae, Morphinae and Satyrinae). Biological Journal of the Linnean Society 92: 467–481. https://doi.org/10.1111/j.1095-8312.2007.00872.x

Butterflies of America (2019) Butterflies of America https://www.butterfliesofamerica.com/L/Nymphalidae.htm [accessed 10. November 2019]

Constantino LM, Salazar JA (2010) A review of the *Philaethria dido* species complex (Lepidoptera: Nymphalidae: Heliconiinae) and description of three new sibling species from Colombia and Venezuela. Zootaxa 2720: 1–27. https://doi.org/10.11646/zootaxa.2720.1.1

de Lesse H (1960) Spéciation et variation chromosomique chez les Lépidoptères Rhopalocères. Annales des Sciences Naturelles Zoologie et Biologie Animale (12e série) 2: 1–223.

de Lesse H (1967) Les nombres de chromosomes chez les Lépidoptères Rhopalocères neéotropicaux. Annales de la Societe Entomologique de France (N.S.) 3: 67–136.

de Lesse H (1970a) Les nombres de chromosomes chez les Lépidoptères Rhopalocères en Amérique centrale et Colombie. Annales de la Societe Entomologique de France (N.S.) 6: 347–358.

de Lesse H (1970b) Formules chromosomiques de quelques Lépidoptères Rhopalocères de Guyane. Annales de la Societe Entomologique de France (N.S.) 6: 849–855.

de Lesse H, Brown Jr KS (1971) Formules chromosomiques de Lépidoptères Rhopalocères du Brésil. Bulletin de la Société entomologique de France 76: 131–137.

de Matos NB, Ferreira M, Silva F, Rodrigues MT, da Silva ES, Garcia C (2016) Taxonomy and evolution of *Tropidurus* (Iguania, Tropiduridae) based on chromosomal and DNA barcoding analysis. Journal of Herpetology 50(2): 316–326. https://doi.org/10.1670/13-221

de-Silva DL, Day JJ, Elias M, Willmott K, Whinnett A, Mallet J (2010) Molecular phylogenetics of the neotropical butterfly subtribe Oleriina (Nymphalidae: Danainae: Ithomiini). Molecular Phylogenetics and Evolution 55: 1032–1041. https://doi.org/10.1016/j.ympev.2010.01.010

deWaard JR, Ivanova NV, Hajibabaei M, Hebert PDN (2008) Assembling DNA barcodes: analytical protocols. In: Martin CC (Ed.) Environmental Genomics, Methods in Molecular Biology. Humana Press, Totowa, 410: 275–283. https://doi.org/10.1007/978-1-59745-548-0_15

Dincă V, Zakharov EV, Hebert PDN, Vila R (2011) Complete DNA barcode reference library for a country's butterfly fauna reveals high performance for temperate Europe. Proceedings of the Royal Society B Biological sciences 278(1704): 347–355. https://doi.org/10.1098/rspb.2010.1089

Garcia-Souto D, Sumner-Hempel A, Fervenza S, Perez-Garcia C, Torreiro A, Gonzalez-Romero R, Eirin-Lopez JM, Moran P, Pasantes JJ (2017) Detection of invasive and cryptic species in marine mussels (Bivalvia, Mytilidae): A chromosomal perspective. Journal for Nature Conservation 39: 58–67. https://doi.org/10.1016/j.jnc.2017.07.005

Hajibabaei M, deWaard JR, Ivanova NV, Ratnasingham S, Dooph RT, Kirk SL, Mackie PM, Hebert PDN (2005) Critical factors for assembling a high volume of DNA barcodes. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 360: 1959–1967. https://doi.org/10.1098/rstb.2005.1727

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences of the United States of America 103: 968–971. https://doi.org/10.1073/pnas.0510466103

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hebert PDN, Gregory TR (2005) The promise of DNA barcoding for taxonomy. Systematic Biology 54: 852–859. https://doi.org/10.1080/10635150500354886

Hebert PDN, Cywinska A, Ball SL, deWaard J (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the United States of America 101(41): 14812–14817. https://doi.org/10.1073/pnas.0406166101

Ivanova NV, deWaard JR, Hebert PDN (2006) An inexpensive, automation-friendly protocol for recovering high quality DNA. Molecular Ecology Resources 6: 998–1002. https://doi.org/10.1111/j.1471-8286.2006.01428.x

Lorković Z (1958) Some peculiarities of spatially and sexually restricted gene exchange in the *Erebia tyndarus* group. Cold Spring Harbor Symposia on Quantitative Biology 23: 319–325. https://doi.org/10.1101/SQB.1958.023.01.032

Lukhtanov VA (2017) A new species of *Melitaea* from Israel, with notes on taxonomy, cytogenetics, phylogeography and interspecific hybridization in the *Melitaea persea* complex (Lepidoptera, Nymphalidae). Comparative Cytogenetics 11(2): 325–357. https://doi.org/10.3897/CompCytogen.v11i2.12370

Lukhtanov VA (2019a) Two types of highly ordered micro- and macrochromosome arrangement in metaphase plates of butterflies (Lepidoptera). Comparative Cytogenetics 13(1): 19–25. https://doi.org/10.3897/CompCytogen.v13i1.32614

Lukhtanov VA (2019b) Species delimitation and analysis of cryptic species diversity in the XXI century. Entomological Review 99(4): 463–472. https://doi.org/10.1134/S0013873819040055

Lukhtanov VA, Dantchenko AV (2017) A new butterfly species from south Russia revealed through chromosomal and molecular analysis of the *Polyommatus* (*Agrodiaetus*) *damonides* complex (Lepidoptera, Lycaenidae). Comparative Cytogenetics 11(4): 769–795. https://doi.org/10.3897/compcytogen.v11i4.20072

Lukhtanov VA, Dantchenko AV, Vishnevskaya MS, Saifitdinova AF (2015) Detecting cryptic species in sympatry and allopatry: analysis of hidden diversity in *Polyommatus* (*Agrodiaetus*) butterflies (Lepidoptera: Lycaenidae). Biological Journal of the Linnean Society 116(2): 468–485. https://doi.org/10.1111/bij.12596

Lukhtanov VA, Dincă V, Talavera G, Vila R (2011) Unprecedented within-species chromosome number cline in the Wood White butterfly *Leptidea sinapis* and its significance for karyotype evolution and speciation. BMC Evolutionary Biology 11: 1–109. https://doi.org/10.1186/1471-2148-11-109

Lukhtanov VA, Shapoval NA (2017) Chromosomal identification of cryptic species sharing their DNA barcodes: *Polyommatus* (*Agrodiaetus*) *antidolus* and *P.* (*A.*) *morgani* in Iran (Lepidoptera, Lycaenidae). Comparative Cytogenetics 11(4): 759–768. https://doi.org/10.3897/compcytogen.v11i4.20876

Lukhtanov VA, Shapoval NA, Dantchenko AV (2014) Taxonomic position of several enigmatic *Polyommatus* (*Agrodiaetus*) species (Lepidoptera, Lycaenidae) from Central and Eastern Iran: insights from molecular and chromosomal data. Comparative Cytogenetics 8(4): 313–322. https://doi.org/10.3897/CompCytogen.v8i4.8939

Lukhtanov VA, Sourakov A, Zakharov EV (2016) DNA barcodes as a tool in biodiversity research: testing pre-existing taxonomic hypotheses in Delphic Apollo butterflies (Lepidoptera, Papilionidae). Systematics and Biodiversity 14(6): 599–613. https://doi.org/10.1080/14772000.2016.1203371

Lukhtanov VA, Sourakov A, Zakharov EV, Hebert PDN (2009) DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. Molecular Ecology Resources 9: 1302–1310. https://doi.org/10.1111/j.1755-0998.2009.02577.x

Maddison WP, Maddison DR (2015) Mesquite: a modular system for evolutionary analysis. Version 3.04. http://www.mesquiteproject.org

Marques DF, dos Santos FA, da Silva SS, Sampaio I, Rodrigues LRR (2015) Cytogenetic and DNA barcoding reveals high divergence within the trahira, *Hoplias malabaricus* (Char-

aciformes: Erythrinidae) from the lower Amazon River. Neotropical Ichthyology 11(2): 459–466. https://doi.org/10.1590/S1679-62252013000200015

McClure M, Dutrillaux B, Dutrillaux A-M, Lukhtanov V, Elias M (2017) Heterozygosity and chain multivalents during meiosis illustrate ongoing evolution as a result of multiple holokinetic chromosome fusions in the genus *Melinaea* (Lepidoptera, Nymphalidae). Cytogenetic and Genome Research 153(4): 213–222. https://doi.org/10.1159/000487107

Pazhenkova EA, Lukhtanov VA (2019) Nuclear genes (but not mitochondrial DNA barcodes) reveal real species: Evidence from the *Brenthis* fritillary butterflies (Lepidoptera, Nymphalidae). Journal of Zoological Systematics and Evolutionary Research 57(2): 298–313. https://doi.org/10.1111/jzs.12252

Petrova NA, Cornette R, Shimura S, Gusev OH, Pemba D, Kikawada T, Zhirov SV, Okuda T (2015) Karyotypical characteristics of two allopatric African populations of anhydrobiotic *Polypedilum* Kieffer, 1912 (Diptera, Chironomidae) originating from Nigeria and Malawi. Comparative Cytogenetics 9(2): 173–188. https://doi.org/10.3897/CompCytogen.v9i2.9104

Przybyłowicz Ł, Lukhtanov V, Lachowska-Cierlik D (2014) Towards the understanding of the origin of the Polish remote population of *Polyommatus* (*Agrodiaetus*) *ripartii* (Lepidoptera: Lycaenidae) based on karyology and molecular phylogeny. Journal of Zoological Systematics and Evolutionary Research 52(1): 44–51. https://doi.org/10.1111/jzs.12040

Robinson R (1971) Lepidoptera Genetics. Pergamon Press, Oxford, 698 pp. https://doi.org/10.1016/B978-0-08-006659-2.50006-7

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Sahoo RK, Warren AD, Wahlberg N, Brower AVZ, Lukhtanov VA, Kodandaramaiah U (2016) Ten genes and two topologies: an exploration of higher relationships in skipper butterflies (Hesperiidae). PeerJ 4: e2653. https://doi.org/10.7717/peerj.2653

Suomalainen E, Brown KS (1984) Chromosome numbers within *Philaethria* butterflies (Lepidoptera: Nymphalidae, Heliconiini). Chromosoma 90: 170–176. https://doi.org/10.1007/BF00292393

Talavera G, Lukhtanov VA, Rieppel L, Pierce NE, Vila R (2013) In the shadow of phylogenetic uncertainty: the recent diversification of *Lysandra* butterflies through chromosomal change. Molecular Phylogenetics and Evolution 69: 469–478. https://doi.org/10.1016/j.ympev.2013.08.004

Tavares JR, de Sousa TP, da Silva JM, Venere PC, Faria KD (2015) Cytogenetics and DNA barcoding of the round-eared bats, *Tonatia* (Chiroptera: Phyllostomidae): a new karyotype for Tonatia bidens. Zoologia 32(5): 371–379. https://doi.org/10.1590/S1984-46702015000500006

Van Nieukerken EJ , Kaila L, Kitching IJ, Kristensen NP, Lees DC, Minet J, Mitter C, Mutanen M, Regier JC, Simonsen TJ, Wahlberg N, Yen S-H, Zahiri R, Adamski D, Baixeras J, Bartsch D, Bengtsson B, Brown JW, Bucheli SR, Davis DR, de Prins J, de Prins W, Epstein ME, Gentili-Poole P, Gielis C, Hättenschwiler P, Hausmann A, Holloway JD, Kallies A,

Karsholt O, Kawahara AY, Koster SJC, Kozlov MV, Lafontaine JD, Lamas G, Landry J-F, Lee S, Nuss M, Park K-T, Penz C, Rota J, Schintlmeister A, Schmidt BC, Sohn J-C, Solis MA, Tarmann GM, Warren AD, Weller S, Yakovlev RV, Zolotuhin VV, Zwick A (2011) Order Lepidoptera Linnaeus, 1758. In: Zhang Z.-Q. (Ed) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa 3148: 212–221. https://doi.org/10.11646/zootaxa.3148.1.41

Vishnevskaya MS, Lukhtanov VA, Dantchenko AV, Saifitdinova AF (2018) Combined analysis of chromosomal and molecular markers reveals cryptic species: karyosystematics of the *Agrodiaetus* Hübner, [1822] blue butterflies. Comparative Cytogenetics 12(3): 325–326. https://doi.org/10.3897/CompCytogen.v12i3.27748

Vishnevskaya MS, Saifitdinova AF, Lukhtanov VA (2016) Karyosystematics and molecular taxonomy of the anomalous blue butterflies (Lepidoptera, Lycaenidae) from the Balkan Peninsula. Comparative Cytogenetics 10(5): 1–85. https://doi.org/10.3897/CompCytogen.v10i5.10944

Ward RD, Hanner R, Hebert PDN (2009) The campaign to DNA barcode all fishes, FISH-BOL. Journal of Fish Biology 74(2): 329–356. https://doi.org/10.1111/j.1095-8649.2008.02080.x

Wesley DJ, Emmel TC (1975) The chromosomes of neotropical butterflies from Trinidad and Tobago. Biotropica 7: 24–31. https://doi.org/10.2307/2989796

White MJD (1973) Animal cytology and evolution. Cambridge, 961 pp.